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| (54) Title: METHOD OF PRODUCING A CHIMERIC PROTEIN | | |
| (57) Abstract A method of producing a chimeric protein from i.e. a plant virus coding for such a protein. The method allows the production of large (i.e. 25 kDa) proteins which assemble with the virus in infected host cells and are arranged on the outer surface of chimeric viruses. A vector for the production of biologically useful proteins in such a manner is also disclosed. | | |

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1 METHOD OF PRODUCING A CHIMERIC PROTEIN

2
3 This invention relates to a method of producing a
4 chimeric protein, eg a biologically active protein such
5 as an antibiotic peptide.

6
7 Typical antibiotic peptides include the marginins, 23
8 amino acid-long alpha-helical peptides, originally
9 identified from frog skin, which have significant
10 antibacterial activity; the defensins which combat
11 bacteria, fungi and some enveloped viruses such as
12 herpes simplex virus and HIV; and the protegrins which
13 are 16-18 amino acid-long antibiotic peptides with
14 strong biocidal activity.

15
16 The protegrins form part of an array of antibiotic
17 peptides that are used by mammalian phagocytes to
18 destroy invading pathogens through non-oxidative
19 processes. Typically the protegrins include 4 cysteine
20 residues and form a double-stranded β -sheet structure
21 and show sequence similarity with the antibiotic
22 defensin peptides that are also involved in phagocyte
23 defence responses. The defensins are cationic,
24 cysteine-rich peptides of 29 to 34 amino acids that are
25 formed almost entirely of β -sheet structures and that

1 have been shown to have biocidal activity against
2 bacteria, fungi and some enveloped viruses, including
3 herpes simple virus and HIV. Both the protegrins and
4 defensins are expressed in phagocytes as pre-pro-
5 proteins which are cleaved to release the biocidal
6 peptides from the carboxy-terminus of the protein.

7
8 Because of their antibacterial activity it may not be
9 convenient to synthesize these antibiotic peptides by
10 genetic engineering in conventional prokaryotic
11 expression systems. Solution synthesis of large
12 amounts of these peptides with a variety of amino acid
13 modifications may be possible, but is not currently
14 considered commercially viable, since a significant
15 drop in yield occurs in the manufacture of peptides of
16 over 25-30 amino acid residues.

17
18 Eukaryotic expression systems (yeast, insect, animal or
19 plant cells which produce foreign proteins or peptides)
20 may be necessary if there is a need for post-
21 translational modification of the desired protein, but
22 fermentation processes for such eukaryotic expression
23 systems are expensive to maintain, provide little
24 flexibility in terms of scaling the process up to
25 industrial production levels and are very susceptible
26 to contamination. Processing and purification of the
27 desired protein can also be complex and costly.

28
29 The use of plants and benign plant viruses offers an
30 opportunity to produce foreign proteins with minimal
31 host cell contamination, thereby reducing contamination
32 problems which could affect successful achievement of
33 the required regulatory body approval for human or
34 veterinary applications.

35
36 It has been proposed in W092/18618 to use plant viruses

1 as vector systems for the expression of foreign
2 nucleotide sequences. WO92/18618 describes the use of
3 a Comovirus (Cowpea Mosaic Virus or CPMV) as an
4 effective vector for such expression and also mentions
5 other spheroidal viruses such as HIV and Picorna-
6 viruses. *Picornaviridae* generally comprise particles
7 of 22-30nm having cubic symmetry; *Comoviridae* have a
8 pair of 28nm particles with a similar symmetry, and HIV
9 is a member of the *Retroviridae* which are generally
10 enveloped 100nm particles containing an icosahedral
11 nucleocapsid.

12
13 One disadvantage of the system disclosed in WO92/18618
14 is that the geometry of the spheroidal viruses
15 precludes large proteins from being produced, since the
16 size and number of chimeric proteins per virus particle
17 (generally 60 for icosahedral virus particles) is
18 limited by the spheroidal geometry of the virus.

19
20 Construction of chimeric proteins in such viruses is
21 also limited to the insertion of the foreign component
22 into a loop in a native virus protein, eg the β -B to β -
23 C loop in VP23 of CPMV, where such insertion does not
24 affect the geometry of the coat protein and/or its
25 ability to self-assemble into a virus particle
26 (virion). As can be appreciated, the size of the
27 peptide which can be tolerated in such an insertion is
28 fairly limited; polypeptides of a maximum of 26 amino
29 acids in length are cited by WO92/18618. Larger
30 polypeptides present in internal insertion sites in
31 coat or capsid proteins of the viruses exemplified may
32 result in disruption of the geometry of the protein
33 and/or its ability to successfully interact with other
34 coat proteins leading to failure of the chimeric virus
35 to assemble. Modified viruses which cannot self-
36 assemble might not infect other host cells and produce

1 whole plant infection. This possible lack of ability
2 to spread the infection of the modified virus
3 constitutes a significant disadvantage in the prior
4 system.

5
6 The present invention contemplates the use of benign
7 high copy number rod-shaped viruses, preferably plant
8 viruses such as potato virus X (PVX), to produce
9 foreign protein connected to viral coat protein
10 subunits. When assembled, the virus particles comprise
11 long helical arrays of more than 1000 identical
12 chimeric proteins (which are typically coat protein -
13 foreign protein fusion molecules) per virion.
14 Generally the foreign protein portion will be displayed
15 on the outer surface of the virus particles.

16
17 A suitable proteolytic degradation site (eg elastase or
18 CNBr) may be engineered into the chimeric protein to
19 permit release of the foreign protein portion from
20 purified virus material. Given the size of the foreign
21 protein and the relevant composition of the possible
22 viruses, it is estimated that between 10 and 30% of the
23 total weight yield of virus particle could comprise the
24 foreign protein. Release of the foreign protein by
25 proteolytic cleavage can be a simple purification
26 regime, followed by removal of the residual innocuous
27 plant virus itself. Yields of plant virus up to 5g per
28 kg wet weight of leaf from potato or tobacco are
29 possible and hence the yields of foreign protein could
30 be very substantial.

31
32 If the foreign protein is left attached to the chimeric
33 protein in the virus particle, the whole virus particle
34 can also be used as a vector for expression and
35 presentation of peptide epitopes for vaccination of
36 animals and/or the delivery of therapeutic single-

1 stranded RNA molecules. This may be of utility in the
2 delivery of anti-sense or triplex nucleotides.

3
4 The present invention provides a method of producing a
5 chimeric protein comprising:

6
7 a. providing a rod-shaped recombinant virus or
8 pseudovirus containing a polynucleotide encoding a
9 chimeric protein having a first (viral) portion
10 and a second (non-viral) portion, the chimeric
11 protein being capable of assembly into a virus
12 particle such that the second portion is disposed
13 on the exterior surface of the assembled virus
14 particle;

15
16 b. infecting a host cell with the virus or
17 pseudovirus; and

18
19 c. allowing replication of the virus or pseudovirus
20 and expression of the chimeric protein in the host
21 cell.

22
23 The term "rod-shaped" as applied herein to viruses
24 includes filamentous or flexuous viruses, which are
25 preferred. It is advantageous to use a virus which is
26 flexuous (ie which can bend easily) since chimeric
27 proteins with large second portions may be able to
28 assemble more easily into virus particles (virions)
29 which are flexuous than those which are rigid. PVX is
30 preferred since it forms a flexuous virion.

31
32 The virus or pseudovirus can preferably assemble in the
33 host cell to produce infective virus particles which
34 comprise nucleic acid and chimeric protein. This
35 enables the infection of adjacent cells by the
36 infective virus or pseudovirus particle and expression

1 of the chimeric protein therein.

2

3 The host cell can be infected initially with virus or
4 pseudovirus in particle form (ie in assembled rods
5 comprising nucleic acid and protein) or alternatively
6 in nucleic acid form (ie RNA such as viral RNA; cDNA or
7 run-off transcripts prepared from cDNA) provided that
8 the virus nucleic acid used for initial infection can
9 replicate and cause production of whole virus particles
10 having the chimeric protein.

11

12 The term "pseudovirus" as used herein means a virus-
13 derived nucleic acid sequence optionally assembled into
14 particles and having an incomplete viral genome as
15 compared to wild-type virus but retaining sufficient
16 viral genes to allow replication and assembly of the
17 pseudovirus. The virus or pseudovirus may contain
18 genetic material foreign to the wild-type virus.

19

20 Optionally, the virus or pseudovirus can be purified
21 from the host cell in order to concentrate the chimeric
22 protein, ie by polyethylene glycol precipitation and/or
23 density gradient centrifugation.

24

25 Optionally, the method may include the step of
26 separating a protein derived from the second portion
27 from the remainder of the chimeric protein after the
28 virus or pseudovirus has been purified from the host
29 cell.

30

31 A linker peptide can be incorporated between the first
32 and second portions and may have the function of
33 spacing the two portions from one another, reducing
34 steric restrictions. Optionally the linker peptide
35 may contain a proteolytic or chemical cleavage site.

36

1 The term "proteolytic or chemical cleavage site" refers
2 to a short sequence of amino acids which is
3 recognisable and subsequently cleavable by a
4 proteolytic enzyme or chemical means. Suitable
5 proteolytic enzymes include trypsin, pepsin, elastase
6 and the like. Alternatively the proteolytic or
7 chemical cleavage site may be a site which is
8 vulnerable to cleavage by other means, for example by
9 addition of chemicals such as cyanogen bromide (CNBr)
10 or acids or by shear. Preferably, the proteolytic or
11 chemical cleavage site is an elastase cleavage site,
12 but other suitable proteolytic cleavage sites can be
13 used with corresponding enzymes.

14
15 The protein derived from the second portion may be
16 separated from the remainder of the chimeric protein
17 before assembly of the virus particle, eg during
18 expression of the genetic material coding for the
19 chimeric protein, or during assembly of the chimeric
20 protein into a virus particle. In this embodiment the
21 host cell will contain free protein derived from the
22 second portion. This embodiment can be useful when
23 expression of very large proteins derived from the
24 second portion is desired. In such an embodiment, the
25 proteolytic or chemical cleavage site may be selected
26 to cleave automatically in a virally-infected host
27 cell.

28
29 The term "proteolytic or chemical cleavage site" may
30 thus also include sequences that cleave automatically
31 such as the FMDV (Foot and Mouth Disease Virus) 2A
32 protease.

33
34 The proteolytic or chemical cleavage site may be an
35 integral part of either the first or second portion.
36 Hence either/or both of the portions may include an

1 integral proteolytic or chemical cleavage site.

2

3 Thus the present invention also provides a method of
4 producing a chimeric protein as defined above, wherein
5 the protein derived from the second portion is purified
6 directly from the host cell after expression.

7

8 The second portion and/or the protein derived therefrom
9 may be relatively large eg over 10kDa. Proteins of 25-
10 30 kDa are suitable for production by the method and
11 even proteins up to 60-70 kDa have been shown to be
12 produced by the method of the invention.

13

14 The first (viral) portion of the chimeric protein may
15 be any protein, polypeptide or parts thereof, derived
16 from a viral source including any genetically modified
17 versions thereof (such as deletions, insertions, amino
18 acid replacements and the like). In certain
19 embodiments the first portion will be derived from a
20 viral coat protein (or a genetically modified version
21 thereof). Mention may be made of the coat protein of
22 Potato Virus X as being suitable for this purpose.
23 Preferably the first portion has the ability to
24 aggregate into particles by first-portion/first portion
25 association. Thus, a chimeric protein molecule can
26 assemble with other chimeric protein molecules or with
27 wild-type coat protein into a chimeric virion.

28

29 In a preferred embodiment of the invention the particle
30 is derived from a potyvirus or even more preferably a
31 potexvirus such as PVX, and in such an embodiment, the
32 second portion is preferably disposed at or adjacent
33 the N-terminus of the coat protein. In PVX, the N-
34 terminus of the coat protein is believed to form a
35 domain on the outside of the virion.

36

1 The second portion of the chimeric protein may be any
2 protein, polypeptide or parts thereof, including any
3 genetically modified versions thereof (such as
4 deletions, insertions, amino acid replacements and the
5 like) derived from a source other than the virus from
6 which the first portion is derived. In certain
7 embodiments the second portion or the protein derived
8 therefrom is a biologically active or useful molecule.
9 The second portion or the protein derived therefrom may
10 also be a diagnostic reagent, an antibiotic or a
11 therapeutic or pharmaceutically active agent.
12 Alternatively the second portion or the protein derived
13 therefrom may be a food supplement.

14
15 In an alternative embodiment, the second portion or the
16 protein derived therefrom may be an indicator protein
17 chosen for its ability to indicate the location of the
18 chimeric protein or of the virus particle. Such an
19 example is the 25kDa jellyfish green fluorescent
20 protein.

21
22 The polynucleotide coding for the second (non-viral)
23 portion may be inserted into an appropriate restriction
24 site in the viral genome. The restriction site adopted
25 for such insertion may be naturally occurring in the
26 viral genome or artificially constructed therein and
27 the polynucleotide coding for the second portion may be
28 ligated therein by conventional means. General
29 techniques for cloning of foreign nucleic acid and
30 construction of chosen restriction sites is
31 comprehensively described in the art and is within the
32 scope of the skilled person.

33
34 It is preferred that the polynucleotide coding for the
35 second portion is inserted at or adjacent a terminus of
36 the polynucleotide coding for the first portion, such

1 that upon translation the chimeric protein has the
2 first portion at one end and the second portion at the
3 opposite end. It is not necessary for the first
4 portion to comprise a whole virus coat protein, but
5 this remains an option.

6
7 The virus particle may be formed by the assembly of
8 chimeric proteins only or by the mixed assembly of
9 chimeric proteins together with some unmodified or less
10 modified forms of the naturally occurring wild-type
11 coat protein which forms the basis of the first
12 portion. For a mixed virus particle of the latter
13 type, there must be present polynucleotide(s) encoding
14 the chimeric protein and the naturally occurring coat
15 protein. The appropriate protein-coding sequences may
16 be arranged in tandem on the same molecule. An
17 alternative would be co-infection (for example of
18 mutually dependant defective viruses or pseudoviruses)
19 of two or more viruses or pseudoviruses, or infection
20 by chimeric virus of a host cell or whole organism
21 (such as a plant) which expresses such a protein
22 intrinsically.

23
24 An advantage is gained by using a virus which forms a
25 particle with a relatively high pitch of helix. PVX
26 has a pitch of 3.4nm and is to be preferred over
27 viruses with a lower pitch. Virus particles with
28 higher pitches may be able to accommodate larger
29 protein insertions on their surfaces since their coat
30 proteins assemble with more space between them than
31 coat proteins of viruses with lower pitches.

32
33 A virus or pseudovirus genetically modified to express
34 the chimeric protein forms a further aspect of the
35 present invention, as does any host cell infected with
36 such a virus or pseudovirus.

1 Preferably, the host cell used to replicate the virus
2 or pseudovirus is a plant cell where the virus is a
3 plant virus, although insect cells, mammalian cells and
4 bacteria can be used with viruses which will replicate
5 in such cells.

6
7 While modifications and improvements may be
8 incorporated without departing from the scope of the
9 invention, embodiments will now be described by way of
10 the following examples and with reference to the
11 accompanying drawings in which:

12
13 Fig 1a shows the structure of a gene for a
14 chimeric protein and of the overcoat vector
15 pTXS.L2a-CP for use in the present invention;
16 Fig 1b is a schematic diagram showing the major
17 features of plasmids useful in the methods of the
18 present invention;

19 Fig 2 shows a western blot of wild type and
20 chimeric protein taken from leaves of a plant
21 infected by a wild-type and a chimeric virus;
22 Fig 3 a, b, c and d show leaves of plants infected
23 with recombinant virus;

24 Fig 4 a, b, c, d and e are micrographs
25 illustrating the subcellular distribution of
26 chimeric protein expressed from chimeric virus
27 nucleic acid;

28 Fig 5 is an electron micrograph showing
29 aggregation and immuno-gold labelling of
30 chimeric viruses;

31 Fig 6 a, b and c are electron micrographs of
32 negatively-stained chimeric viruses; and

33 Fig 7 is a photograph of a *N benthamiana* leaf
34 systemically infected with a chimeric virus.

35
36 EXAMPLE 1

1 A general strategy for the production of large
2 quantities of recombinant proteins is given below using
3 PVX as an example. A similar strategy could be
4 employed for other flexuous filamentous or rod-shaped
5 viruses. A cDNA clone of potato virus X is first
6 modified to produce fusion proteins between the viral
7 coat protein and proteins with biological activity or
8 other commercial applications. The feasibility of this
9 approach has been demonstrated as described below by
10 creating a translational fusion between the green
11 fluorescent protein (25 kDa) of *Aequorea victoria* (1)
12 and the PVX coat protein (also around 25 kDa).
13 Functional chimeric viruses have also been made which
14 are able to express recombinant genes encoding fusions
15 between the PVX coat protein and the kanamycin
16 resistance protein Neomycin phosphotransferase (25 kDa)
17 and between PVX coat protein and the more complex
18 enzymes β -galactosidase (10-13 kDa) and β -glucuronidase
19 (68 kDa) respectively.

20

21 The green fluorescent protein (GFP) from *A. victoria*
22 (1) is a reporter of gene expression in heterologous
23 systems (3-6). GFP has an advantage over other marker
24 proteins in that it can be detected non-invasively,
25 without any requirement for exogenous substrates or co-
26 factors (3) since it fluoresces intrinsically without a
27 requirement for exogenous substrate. In addition,
28 fluorescence of GFP is retained in fusion proteins
29 allowing the subcellular localization of fusion
30 proteins (4).

31

32 PCR-mutagenesis of a full-length cDNA copy of the
33 potato virus X genome can be performed to create a
34 synthetic coding sequence comprising the gene coding
35 for the protein of interest, the foot and mouth disease
36 virus 2A protease gene, and the potato virus X coat

1 protein gene. The PVX genome is contained within the
2 known plasmid pTXS (Fig. 1, reference 25).

3
4 When reassembled the modified cDNA copy of the viral
5 genome can be used as a template to synthesize *in vitro*
6 run-off transcripts. Inoculation of transcripts to
7 plants can be performed by manual abrasion of
8 carborundum coated leaves of either *Nicotiana*
9 *clevelandii* or *N benthamiana*.

10
11 When the above approach was followed using PVX modified
12 to express GFP-CP fusion protein, between two and three
13 days post inoculation the presence of fluorescent
14 regions in the virus infected plants could be observed
15 by eye on inoculated leaves by viewing plants under
16 ultraviolet light. At about ten days post inoculation
17 GFP-mediated fluorescence was detected in systemic
18 (non-inoculated) leaf tissue (Figure 7). This
19 fluorescence was specific to the green fluorescent
20 protein and was not observed on control plants
21 inoculated with wild-type PVX.

22
23 Electron microscopic analysis of viral particles showed
24 a clear increase in particle width in plants infected
25 with the GFP-CP containing virus compared with
26 particles isolated from plants infected with wild-type
27 PVX (Figure 6).

28
29 In the strategy used above, foreign proteins were
30 expressed by fusing them to the amino-terminus of the
31 PVX coat protein. However other sites may be possible,
32 eg carboxy-terminus surface loops on some other rod-
33 shaped or filamentous viruses.

34
35 Data from previous studies suggest that fusion of the
36 proteins to the amino terminus of the PVX coat protein

1 is most likely to be successful. Biochemical,
2 immunological and tritium bombardment data suggest a
3 model for the structure of the PVX coat protein (10) in
4 which the N-terminal 33 amino acids form a domain of β -
5 sheet on the outside of the virion. In contrast, the
6 C-terminus of the PVX coat protein, which also forms
7 part of a β -sheet structure, is inaccessible from the
8 outside of the virion and deletions within it do not
9 permit the virus to infect plants systemically.

10
11 As an additional optional strategy, the foot and mouth
12 disease virus (FMDV) 2A protease sequence (12) can be
13 positioned between the foreign and coat protein
14 sequences. The FMDV 2A protease is a short (19 amino
15 acid) peptide which acts *in cis* to cleave the FMDV
16 polyprotein in a co-translational mechanism. This
17 protease has been shown to effect the cleavage of
18 synthetic polyproteins both *in vitro* and *in vivo* (13).
19 The inclusion of the 2A protease sequence between the
20 GFP and coat protein can generate a mixed pool of
21 fusion and cleaved proteins in virus infected cells.
22 The presence of free coat protein, generated by 2A
23 protease mediated cleavage, may circumvent this problem
24 by allowing assembly of virions composed of both free
25 (ie cleaved) and fused coat protein subunits.

26
27 The formation of virions is an absolute requirement of
28 PVX for systemic infection of plants (15). The
29 demonstration herein that GFP-coat protein fusions do
30 assemble into virions (Fig 7) and spread indicates that
31 the size of GFP (25kDa) does not interfere with virion
32 assembly. Fusion proteins which fail to assemble due
33 to size or other constraints can be produced in
34 constructs carrying the FMDV 2A protease, or in plants
35 which are modified to express wild-type coat protein
36 for the particular virus used. The sequence of the 2A

1 protease peptide can be modified to increase or
2 decrease the efficiency of co-translational cleavage.

3
4 EXAMPLE 2

5 This example describes a modified form of PVX which
6 expresses a chimeric gene encoding a fusion between the
7 *Aequorea victoria* green fluorescent protein and the PVX
8 coat protein and assembles into virions that are over
9 twice the diameter of wild-type PVX. The modified
10 virus moves from cell-to-cell and systemically. The
11 example demonstrates the potential of fusions between
12 non-viral protein and virus coat protein for production
13 of high levels of non-viral proteins in plants.

14
15 The plasmids used in this work were derived essentially
16 from the plasmid pTXS which contains the PVX genome and
17 a T7 promoter (described in 25). Fig 1b shows the
18 following main features of the plasmids: the virus RNA-
19 dependent RNA polymerase gene (RdRp); virus genes
20 encoding movement proteins (M1, M2, M3); the virus coat
21 protein gene (CP); promoters from T7 bacteriophage (T7)
22 or for the 35S RNA of CaMV (CaMV35S); the
23 transcriptional terminator of the nopaline synthase
24 gene of *Agrobacterium tumefaciens* and various
25 restriction enzyme sites.

26
27 The plasmid pCXA3 was constructed by transfer of the
28 PVX cDNA from pTXS into the plasmid pB1220.5 between
29 the CaMV 35SRNA promoter and the nopaline synthase gene
30 terminator. The plasmid pB1220.5 is similar to the
31 plasmid pB1221.1 but without the GUS gene (described in
32 27). The junction between the promoter and the PVX
33 cDNA was modified by oligonucleotide directed
34 mutagenesis to the sequence
35 (5')gatttgagagga*gaaaactaaacca(3') in which * denotes
36 the most 3' non-transcribed position in the promoter

1 sequence and the most 5' transcribed position in the
2 viral genome (28). Construction of the pVX201 vector
3 from pCXA3 and pPC2S exploited unique restriction sites
4 at positions 4945 (Apa1) and 6302 (Xho1) of the PVX
5 cDNA (25).

6
7 GFP cDNA was PCR-amplified with primers
8 (5')gccaattcgatcatgagtaaaggag(3') on the positive strand
9 and (5')ggaagtcgacacattttatttg(3') from the negative
10 strand. The bold type represents the initiation and
11 termination codons of the GFP gene (29). The
12 underlined type represents Cla1 and Sal1 sites used to
13 introduce the PCT-amplified sequence into pPVX201 to
14 generate pPVX204. The plasmid pTXS.GFP was made by
15 substitution of the region of pPVX204 containing the
16 GFP sequence into the homologous region of pPC2S.

17
18 The plasmid pTXS.GFP carries a full-length cDNA copy of
19 the potato virus X (PVX) genome into which the GFP gene
20 has been inserted. Inoculation of plants with
21 transcripts synthesized *in vitro* from pTXS.GFP results
22 in the expression of free GFP in infected cells (5).
23 We prepared a derivative of pTXS.GFP, pTXS.GFP-CP, to
24 create a translational fusion between the
25 carboxyterminus of the GFP and the amino-terminus of
26 the PVX coat protein (CP). pTXS.GFP was used as a
27 template to produce the GFP-2A-CP fusion gene by
28 overlap extension PCR using flanking oligonucleotides
29 complementary to the PVX genome and mutagenic
30 oligonucleotides to incorporate the 2A protease coding
31 sequence. Amplified product was subcloned into
32 pTXS.GFP as a 1.5 kbp fragment using the unique
33 restriction sites Cla1 and Xho1 to give pTXS.GFP-CP.
34 Fig. 1a shows a schematic representation of viral cDNAs
35 used to synthesize infectious run-off transcripts for
36 the GFP-2A-CP fusion gene. The predicted Mrs of the

four viral proteins common to all constructs are indicated (K=kD). The polypeptide chain lengths of the CP, GFP and 2A protease (2A) enclosed by the constructs are shown. The bars indicate the position of the subgenomic promoter for the CP. TXS=wild-type PVX; TXS.GFP=PVX modified to express free GFP from a duplicated subgenomic promoter; TXS.GFP-CP=PVX modified to express the GFP-2A-CP fusion protein.

Because the GFP and PVX CP are of similar sizes, having molecular weights of 26.9 kD and 25.1 kD respectively, it was expected that in a homogenous population of fusion protein steric effects would prevent virion formation. Assembly of fusion protein into virions might be facilitated by the presence of a pool of free CP. Therefore the GFP and CP nucleotide sequences in pTXS.GFP-CP were separated by sequence coding for sixteen amino acids from the foot-and-mouth disease virus (FMDV) 2A peptide. The 2A region of FMDV mediates a primary (co-translational) processing event between the 2A and 2B regions of the FMDV polyprotein (12) that results in inhibition of peptide bond formation (13).

In vitro run-off transcripts (14), synthesized from pTXS.GFP and pTXS.GFP-CP (plasmids were linearized with Spe 1 prior to in vitro transcription reactions as described in reference 14), were infectious when inoculated to plants; virus derived from transcript-infected plants is subsequently referred to as PVX.GFP and PVX.GFP-CP respectively.

Following inoculation of either *Nicotiana clevelandii* or *N. benthamiana*, both PVX.GFP and PVX.GFP-CP caused the development of green fluorescent regions which were first detectable by eye under UV illumination between

1 two and three days post inoculation (Fig. 3A, C).
2 Subsequent long-distance movement of the virus to
3 developing leaves led to the appearance of green
4 fluorescence in systemically infected leaves (Fig. 3B,
5 D). The rate at which fluorescent regions spread on
6 inoculated leaves was slower in PVX.GFP-CP infected
7 plants than PVX.GFP infected plants and the appearance
8 of fluorescence in systemically infected leaves was
9 delayed in plants infected with PVX.GFP-CP compared
10 with PVX.GFP infected plants.

11
12 Fig. 3 shows leaves of *N. benthamiana* infected with
13 either PVX.GFP (A, B) or PVX.GFP-CP (C,D). Leaves were
14 viewed under UV illumination (365 nm) generated from a
15 Blak Ray B100-AP lamp (Ultra-Violet Products) and
16 photographed using a Wratten 58 filter to eliminate
17 chlorophyll auto-fluorescence. The pattern of virus
18 spread in both cases is similar. A and C identify
19 inoculated leaves showing the development of
20 characteristic circular legions. B and D identify
21 systemically infected leaves showing fluorescence
22 associated predominantly with the leaf veins. The
23 developing leaf (D) was undergoing the sink-source
24 transition (20) resulting in lack of virus movement
25 into the apical portion of the leaf.

26
27 Fig 4a is a confocal fluorescence image of a
28 systemically infected leaf in transverse section
29 showing the location of PVX.GFP-CP containing
30 viroplasms within individual cells of the leaf. 4b is
31 a bright field image of section shown in (A) showing
32 the typical arrangement of epidermis (E), palisade (P)
33 and mesophyll(M) cells. A vascular bundle (B) is also
34 present (scale=50 μ m). 4c is a confocal image of
35 palisade cells from a leaf systemically infected with
36 PVX.GFP-CP showing the GFP-containing viroplasms (V)

1 assembled into cage-like structures (scale=5 μ m). 4d
2 shows a leaf trichome systemically infected with
3 PVX.GFP, in which the GFP is associated with the
4 nucleus (N) and the cytoplasm. 4e shows a leaf
5 trichome systemically infected with PVX.GFP-CP, in
6 which the GFP is predominantly targeted to viroplasms
7 (V) within individual trichome cells (scale=10 μ m).
8

9 In systemically infected (ie non-inoculated) leaves
10 both PVX.GFP and PVX.GFP-CP moved from the phloem into
11 surrounding bundle sheath and mesophyll cells and
12 eventually into the epidermis (Fig. 4A, B). Under the
13 confocal microscope transverse sections of the
14 systemically infected leaves showed that in PVX.GFP-CP
15 infected cells green fluorescence was detected
16 predominantly in viroplasms, cytoplasmic structures
17 comprising aggregated viral particles that often
18 appeared as continuous cage-like structures within the
19 cell (Fig 4C, 5). By contrast, in PVX.GFP infected
20 cells, the green fluorescence was associated with
21 nuclei and showed a relatively uniform distribution
22 throughout the cytoplasm. This difference in the
23 subcellular distribution of the GFP was seen clearly in
24 leaf trichome cells (Fig. 4D, E).
25

26 The distribution of fluorescence suggested that the
27 majority of GFP produced in PVC.GFP-CP infected plants
28 was still fused to the CP and that these fusion
29 proteins were assembling into virions, which
30 subsequently formed viroplasms.
31

32 Western blotting of protein extracts from inoculated *N.*
33 *clevelandii* leaves, probed with CP specific antiserum
34 (16), showed that most of the immunoreactive protein in
35 PVX.GFP-CP infected plants comprised the fusion
36 protein. Protein extracts were prepared by grinding

1 leaf tissue in two volumes (w/v) protein extraction
2 buffer (15). An equal volume of 2x SDS load buffer was
3 added and the extracts were boiled for two minutes.
4 Proteins were electrophoresed, blotted to
5 nitrocellulose and probed with rabbit polyclonal anti-
6 PVX CP antiserum as described previously (16). Fig 2
7 illustrates the data obtained. Protein was prepared
8 from mock inoculated control plants (lane 2), or from
9 plants inoculated with in vitro transcripts synthesized
10 from plasmid DNAs (TXS=lane 1; TXS.GFP-CP=lane 3;
11 TXS.GFP=lane 4). Mrs of native CP, the GFP-2A-CP
12 fusion protein and CP released by 2A protease mediated
13 cleavage are 25.1, 53.2 and 24.8 kD respectively. The
14 Mrs of standards are shown to the left of Fig 2 in kD.
15
16 The low level of smaller immunoreactive protein
17 detected in PVX.GFP-CP infected tissue is assumed to
18 result from processing of the fusion protein mediated
19 by the FMDV 2A peptide rather than from contamination
20 with virus deletion mutants as similar ratios of fusion
21 to free protein were observed in all other samples
22 analyzed and RT-PCR analysis of the same samples used
23 for protein analysis showed no evidence of deleted
24 forms of the viral genome (17). In addition when blots
25 were probed with GFP specific antiserum the ratio of
26 free protein to fusion protein was the same as that
27 observed using anti-CP antiserum (17).
28
29 In order to determine the subcellular location of the
30 viral CP ultrathin sections of inoculated leaves were
31 prepared for immuno-gold labelling, using a polyclonal
32 antibody to the PVX CP. Leaf tissues were fixed and
33 embedded in Araldite (TM) resin for immuno-gold
34 labelling as described previously (17). Ultrathin
35 sections on nickel grids were labelled using polyclonal
36 rabbit antiserum to the PVX CP followed by goat anti-

1 rabbit gold conjugate (GAR-15 nm, Amersham
2 International). Aggregation of the filamentous
3 virions into viroplasms is marked with arrows in Fig 5.
4 Dense gold labelling was predominantly associated with
5 the viroplasms in both PVX.GFP and PVX.GFP-CP infected
6 cells. The pattern of virus aggregation seen in the
7 electron microscope for both PVX.GFP-CP (Fig. 5) and
8 PVX.GFP was remarkably similar to the cages of
9 viroplasm seen with PVX.GFP-CP under the confocal
10 microscope (Fig. 4c).

11
12 For negative staining, virus particles were trapped
13 from virus infected sap extracts by immuno-sorbent
14 electron microscopy (18) using anti-PVX CP antiserum,
15 and stained with 2% sodium phosphotungstate (pH 7).
16 Analysis of negatively stained virus samples under the
17 electron microscope revealed that PVX.GFP-CP virions
18 were decorated along their length with globular
19 extensions (Fig. 6a,b). Fig 6c shows negatively
20 stained virus rods isolated from PVX.GFP infected
21 plants (scale=50 nm). Differences in virion diameter
22 are seen most clearly where virions are aligned in
23 parallel (a and c, large darts). In Fig 6b small
24 globular extensions (small darts) are apparent along
25 the length of the PVX.GFP-CP virus (scale=25 nm). The
26 PVX.GFP-CP virions had a mean diameter of 29.7 nm, more
27 than twice the diameter of PVX.GFP virions (12.6 nm;
28 Fig. 6c).

29
30 A modified form of PVX.GFP-CP, in which the FMDV 2A
31 peptide sequence carries three amino acid
32 substitutions, introduced to prevent processing of the
33 polyprotein, was unable to move from cell-to-cell and
34 did not give rise to fluorescent viroplasms.
35 Infections with this mutant were restricted to single
36 epidermal cells and fluorescence was detected uniformly

1 throughout the cytoplasm and in association with
2 nuclei, as observed for PVX.GFP infections (17),
3 suggesting that the presence of free CP is essential
4 for either initiation of elongation of virions.

5
6 The fluorescence generated by the GFP attached to
7 virions was intense, allowing rapid detection of viral
8 aggregates within individual living cells.
9 Furthermore, confocal microscopy allowed the
10 noninvasive imaging of the pathway of cell-to-cell
11 movement of virus-GFP constructs, pinpointing the
12 specific cell types in which virus accumulated. For
13 confocal imaging leaves were excised from the plant and
14 sectioned transversely into 200 μ m slices using a
15 vibrotome. The sections were immediately mounted in
16 water and viewed under a Bio-Rad MRC 1000 confocal
17 laser scanning microscope at an excitation wavelength
18 of 488 nm using a krypton-argon laser.

19
20 Previous descriptions of assembly competent plant RNA
21 viruses carrying CP extensions have involved small
22 oligopeptide fusions (19). The data presented in this
23 example suggest that the system described could be used
24 for the production of proteins that are at least as
25 large as the viral CP of PVX.

26
27 The strategy described to generate GFP-coat protein
28 fusions can be easily applied to proteins other than
29 GFP. We modified the plasmid pTXS.GFP-CP which carries
30 the GFP-2A-CP fusion protein gene to enable the facile
31 insertion of novel coding sequence as a fusion to the
32 2A-CP cassette. This modified plasmid, pTXS.L2a-CP
33 shown in Fig. 1a (deposited under No NCTC 12918 at the
34 National Collection of Type Cultures at 61 Colindale
35 Avenue, London NW9 5HT on 18 October 1995) carries a
36 series of unique restriction enzyme recognition sites

(Cla1, Eag1, Sma1, Ehe1) or polylinker that replaces the GFP coding sequence of pTXS.GFP-CP. By digesting the vector pTXS.L2a-CP at one or more of the polylinker restriction enzyme sites it is possible to insert the coding sequence for any given protein such that a fusion protein gene is created comprising the novel gene, the FMDV 2A peptide and the PVX coat protein as a translational fusion.

The plasmid vector pTXS.L2a-CP was prepared by PCT-based mutagenesis of the plasmid pTXS.GFP-CP using standard techniques (26). The oligonucleotide 2aL5' was annealed to the primer 2aL3' and extended with T4 DNA polymerase.

The sequence of primers used was

2aL5': 5' TCG GCC GTC CCG GGG GCG 3'

||| ||| ||| ||| ||| |||

2aL3': 3' AGC CGG CAG GGC CCC CGC GGT TAA AAC TGG AAG

AAT TCG AAA 5'

The extended product was gel purified and cloned into the plasmid M13RK8.2 (30). An Eag 1/ Afl 11 fragment was excised from the resulting plasmid and cloned between the same sites of the plasmid pTXS.GFP-CP in place of the GFP gene.

Thus, the nucleotide sequence of the new linker in pTXS.L2a-CP is

Cla1 Eag1 Sma1 Ehe1

/____\ /____\ /____\ /____\

Nts: AT CGA TCC GGC CGT CCC GGG GGC GCC AAT TTT

Amino acids: Pro Gly Gly Ala Asn Phe

1 Insertion of foreign genes into the pTXS.P-CP
2 polylinker are most easily performed by PCR
3 amplification of the foreign gene using
4 oligonucleotides designed to incorporate appropriate
5 restriction enzyme recognition sites at the 5'- and 3'-
6 termini of the foreign coding sequence such that the
7 gene for the synthetic polyprotein comprises a single
8 open reading frame. We have demonstrated the utility
9 of this approach using the gene encoding neomycin
10 phosphotransferase (NPT) which confers resistance to
11 the antibiotic kanamycin and is present in most
12 commercially available plasmids as a selection tool.
13 The 0.73 kb (NPT) coding sequence was inserted into the
14 polylinker of pTXS.P-CP to give the plasmid pTXS.NPT-
15 CP. Transcripts synthesized in vitro from the
16 pTXS.NPT-CP template were infectious on plants and the
17 virus moved both locally and systemically. Assembly of
18 PVX.NPT-CP virions results in "overcoat" virus
19 particles carrying the NPT protein on the surface of
20 the virions.

21

22 The advantages of the invention are as follows:

23

24 (i) Standard purification procedures exist (eg
25 polyethylene glycol precipitation and centrifugation)
26 for these highly stable virus particles to remove plant
27 proteins and cellular debris and to give an extremely
28 pure suspension of plant virus particles. Plant
29 viruses are innocuous to humans, ingestion experiments
30 have already revealed that they pass straight through
31 the intestine undamaged.

32

33 (ii) By attaching the foreign protein to each (or a
34 subset of) coat protein subunits optionally with a
35 suitable cleavage-sensitive linker sequence will allow,
36 following virus purification from the infected plant

1 sap, foreign protein to be released into free solution
2 simply by incubation with the appropriate proteolytic
3 enzyme. The released virus particles remain stable and
4 of high molecular weight so that they can be separated
5 from the short peptide either by simple dialysis
6 procedures (continuous flow type), or by differential
7 centrifugation or selective precipitation.

8
9 (iii) Yields of cleaved foreign protein from such a
10 system could reach 50% or more of the total weight of
11 virus recovered. Each helical virus particle has 95%
12 of its weight as coat protein, and each coat protein
13 subunit has a molecular weight of approximately 25 kD.
14 In the model system already developed the green
15 fluorescent protein also has a molecular mass of
16 approximately 25 kD. Yields of potato virus X can be
17 extremely high (up to 5 gm/kg wet weight of infected
18 leaf after several weeks).

19
20 (iv) The flexibility of scale that can be achieved in
21 plants is also attractive in terms of reducing the cost
22 of protein production and avoids the need for high
23 level capital investment such as in animal or microbial
24 cell culture facilities.

25
26 (v) The use of set-aside land and/or discredited crops
27 such as tobacco for the alternative production of
28 highly prized, pharmaceutically active proteins would
29 lead to considerable added value in the peri-
30 agricultural sector.

31

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3 The following documents referred to in the text are
4 incorporated herein by reference:

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1 Claims:

2

3 1 A method of producing a chimeric protein, the
4 method comprising:

5

6 a providing a rod-shaped recombinant virus or
7 pseudovirus containing a polynucleotide encoding a
8 chimeric protein having a first (viral) portion
9 and a second (non-viral) portion, the chimeric
10 protein being capable of assembly into a virus
11 particle such that the second portion is disposed
12 on the exterior surface of the assembled virus
13 particle;

14

15 b infecting a host cell with the virus or
16 pseudovirus; and

17

18 c allowing replication of the virus or pseudovirus
19 and expression of the chimeric protein in the host
20 cell.

21

22 2 A method according to claim 1, wherein the
23 chimeric protein assembles into a virus particle.

24

25 3 A method according to claim 1 or claim 2, wherein
26 the virus or pseudovirus is subsequently purified from
27 the host cell.

28

29 4 A method according to claim 2 or claim 3,
30 including the step of cleaving the second portion or a
31 protein derived therefrom from the first portion after
32 purification of the virus or pseudovirus from the host
33 cell.

34

35 5 A method according to any preceding claim, wherein

36

1 a linker peptide is incorporated between the first and
2 second portions.

3

4 6 A method according to any preceding claim, wherein
5 a proteolytic cleavage site is incorporated on one of
6 or between the first and second portions.

7

8 7 A method according to claim 1, wherein the first
9 and second portions are separated from one another
10 before or during assembly of the virus particle, such
11 that the host cell contains free protein derived from
12 the second portion.

13

14 8 A method according to any preceding claim, wherein
15 protein derived from the second portion is purified
16 from the host cell after replication.

17

18 9 A method according to any preceding claim, wherein
19 the virus or pseudovirus is derived from a plant virus.

20

21 10 A method according to any preceding claim, wherein
22 the virus or pseudovirus is derived from potato virus
23 X.

24

25 11 A method according to any preceding claim, wherein
26 the second portion is disposed at or adjacent the N-
27 terminus of the viral coat protein.

28

29 12 A method according to any preceding claim, wherein
30 the second portion is a diagnostic reagent, an
31 antibiotic, a therapeutic or pharmaceutically active
32 agent, a vaccine or a food supplement.

33

34 13 A method according to any preceding claim, wherein
35 the virus or pseudovirus particle comprises a mixture
36 of chimeric protein and wild-type coat protein.

1 14 A method according to any preceding claim, wherein
2 the virus or pseudovirus particle has a relatively high
3 pitch of helix.

4
5 15 A method according to claim 12, wherein the pitch
6 of the helix is more than 2nm.

7
8 16 A method according to any preceding claim, wherein
9 the virus or pseudovirus is flexuous.

10
11 17 A method according to any preceding claim, wherein
12 the host cell is infected with virus or pseudovirus in
13 particle form.

14
15 18 A method according to any one of claims 1-16,
16 wherein the host cell is infected with virus or
17 pseudovirus in nucleic acid form.

18
19 19 A method according to any preceding claim, wherein
20 the second portion or a peptide derived therefrom has a
21 molecular weight in excess of 10 kDa.

22
23 20 A virus or pseudovirus genetically modified to
24 express a chimeric protein, the chimeric protein having
25 a first (viral) portion linked to a second (non-viral)
26 portion, the chimeric protein being capable of self-
27 assembly into a virus particle so that the second
28 portion is disposed on the exterior surface of the
29 assembled virus particle.

30
31 21 A host cell, plant, animal or insect infected
32 with a virus or pseudovirus according to claim 20.

33
34 22 A polynucleotide capable of producing a virus or
35 pseudovirus according to claim 20.

36

1 23 A chimeric protein produced by a method according
2 to any one of claims 1-19.

3

4 24 The plasmid pTXS.L2a-CP as deposited under No NCTC
5 12918 on 18 October 1995 at the National Collection of
6 Type Cultures.

7

8

9

FIGURE 1a

1/7

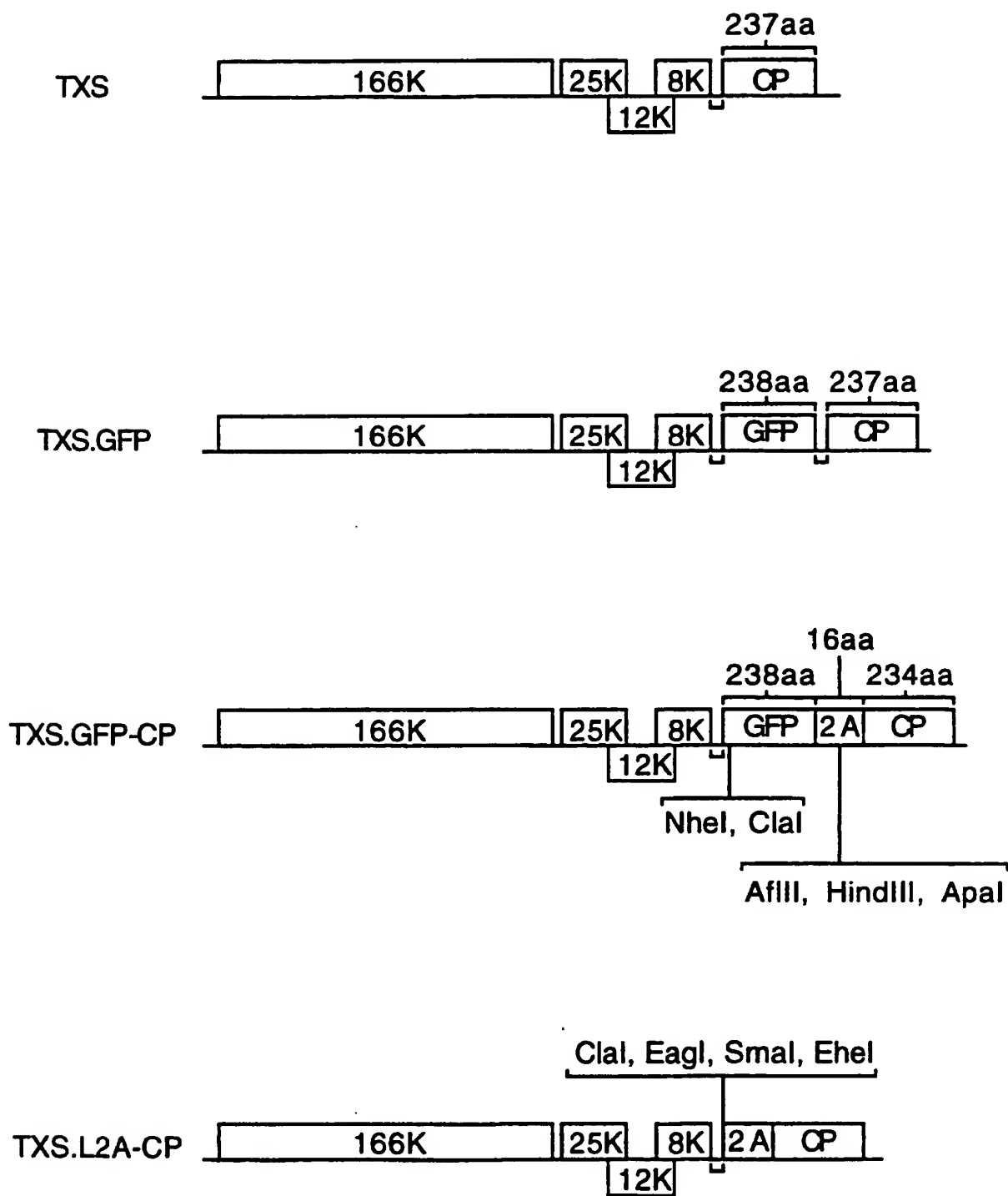
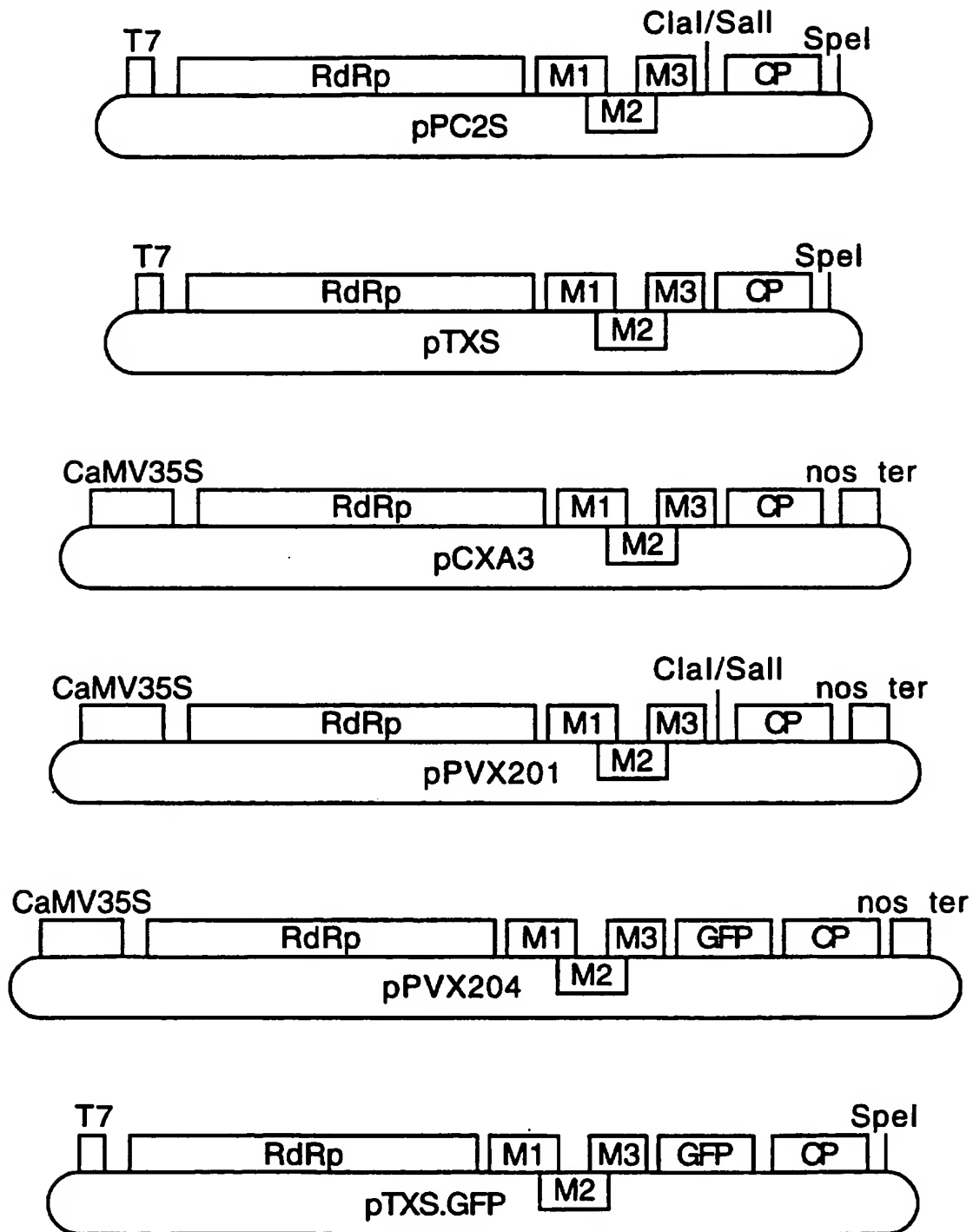


FIGURE 1b

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FIGURE 2

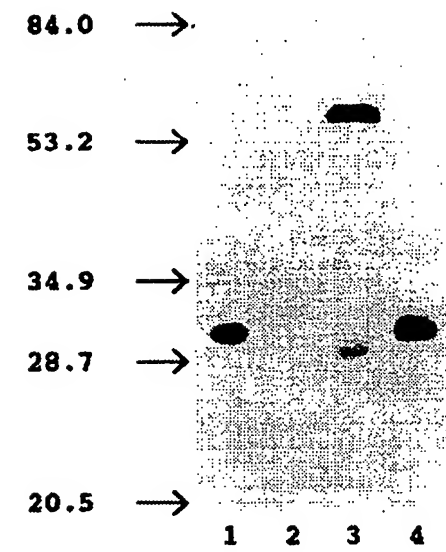


FIGURE 3

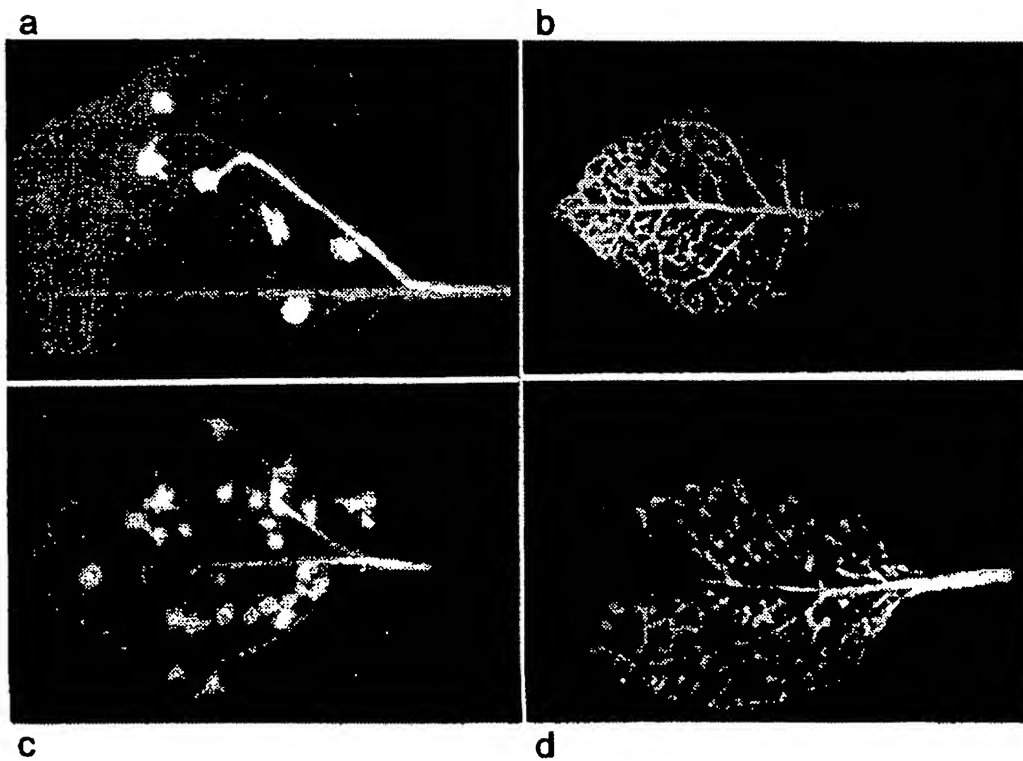
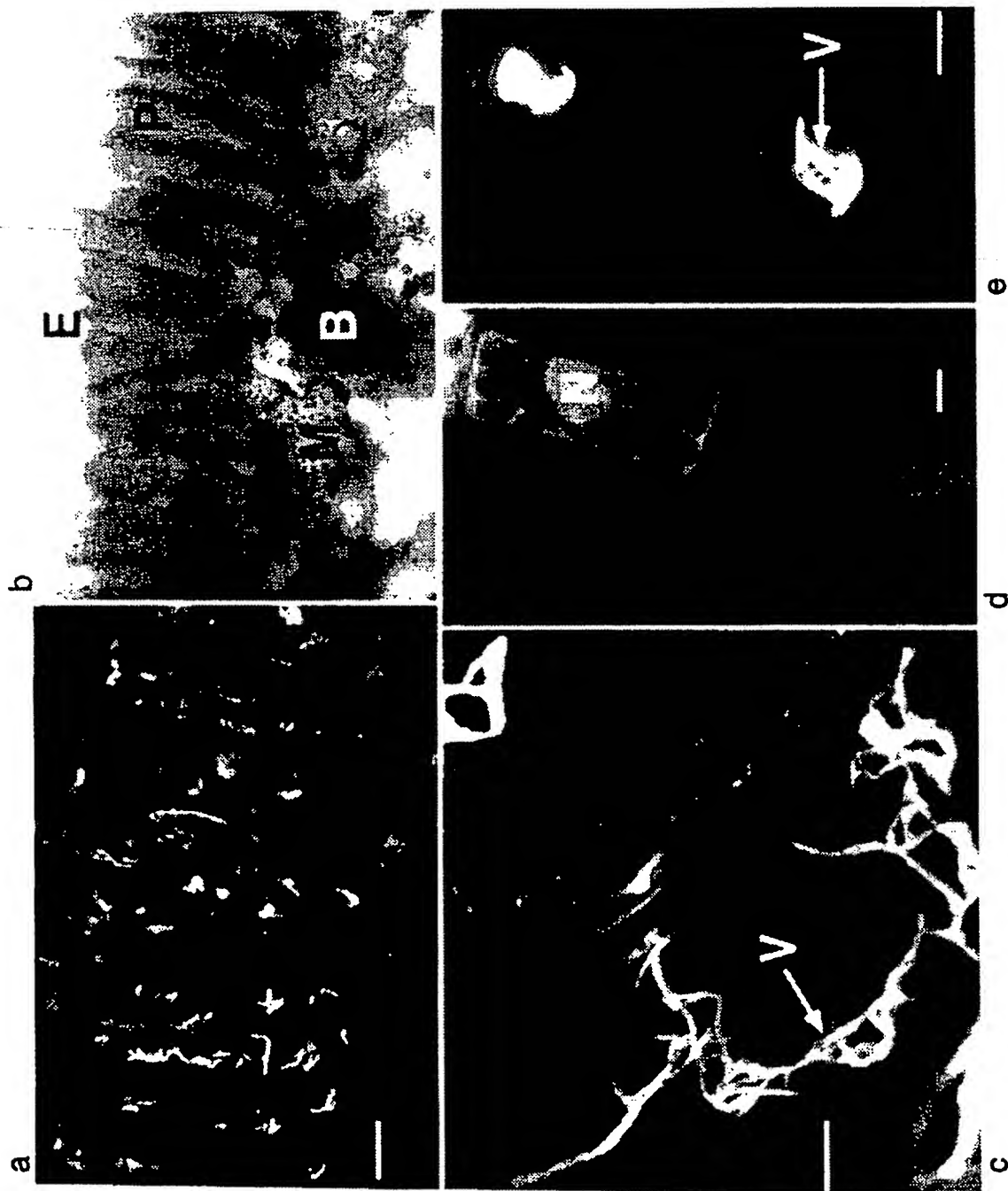


FIGURE 4

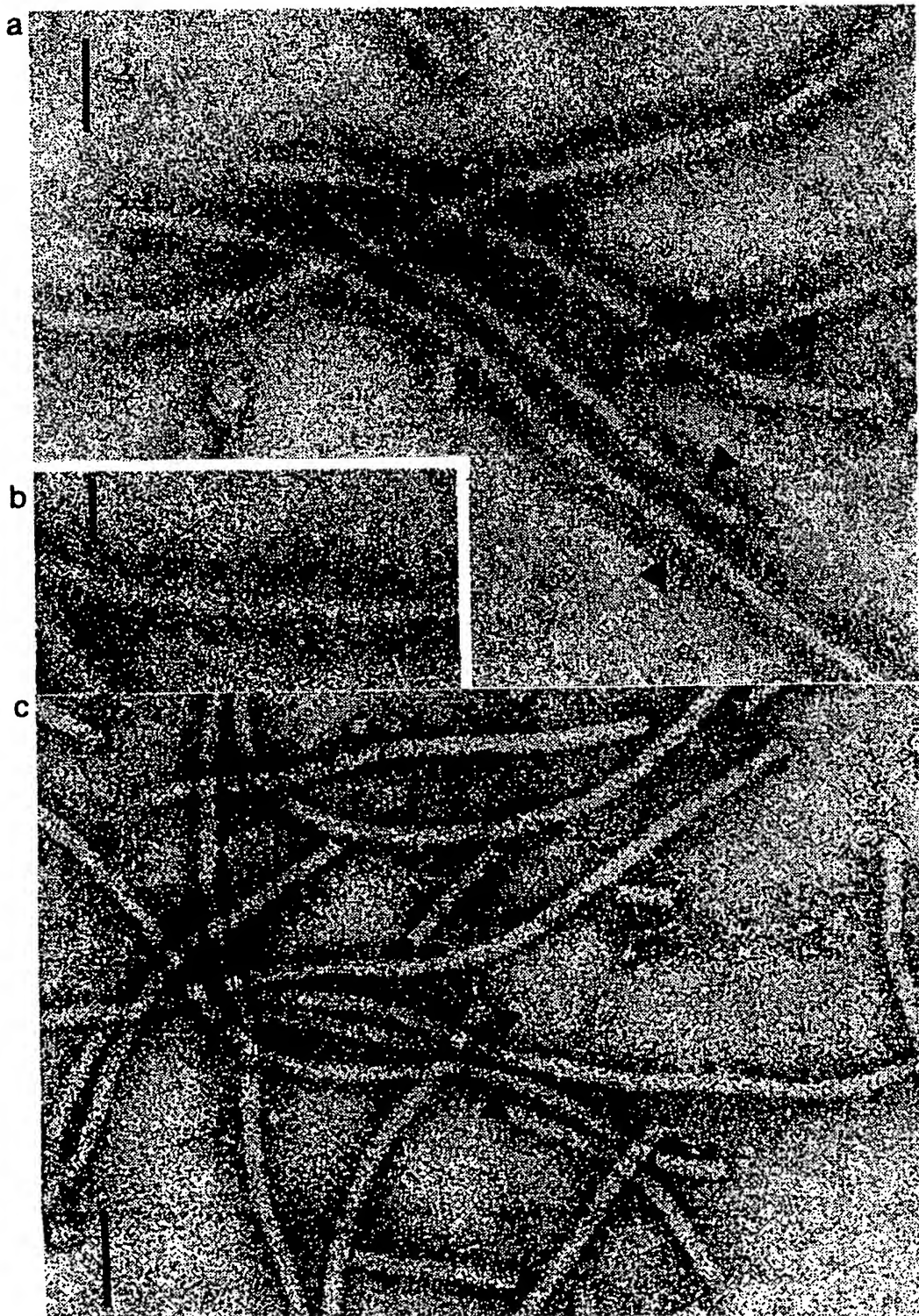


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FIGURE 5

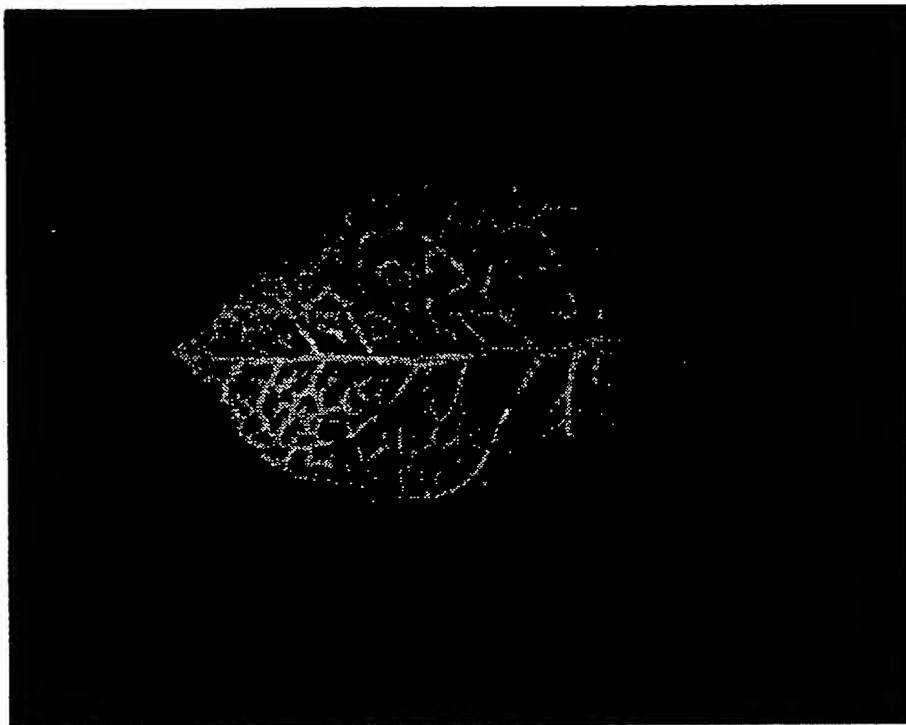


FIGURE 6



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FIGURE 7



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 95/02457

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C12N15/83 C07K14/08 C07K14/435 C12N7/01
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|--------------------------------------|
| X | <p>WO,A,91 15587 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 17 October 1991 see page 7, line 8 - line 22; example 4 see page 3, line 18 - page 4, line 27 --- -/--</p> | <p>1-3,8,9, 12,18, 20-23</p> |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

26 January 1996

Date of mailing of the international search report

15.03.1996

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Montero Lopez, B

INTERNATIONAL SEARCH REPORT

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| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | <p>BIOTECHNOLOGY, vol. 11, no. 10, October 1993 NEW YORK US, pages 1166-1170, MITTUR N. JAGADISH ET AL. 'High level production of hybrid Potyvirus-like particles carrying repetitive copies of foreign antigens in Escherichia coli' see abstract see page 1166, left column, paragraph 3 - page 1167, right column, paragraph 1 see page 1167, right column, paragraph 4 - page 1169, left column, paragraph 3 ---</p> | <p>1-3, 9-13, 20-23</p> |
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| X | <p>DATABASE WPI Section Ch, Week 9013 Derwent Publications Ltd., London, GB; Class B04, AN 90-096520 'New plant virus RNA vector obtained by connecting foreign gene downstream of coat protein gene of tobamo virus RNA' & JP,A,02 049 591 (SUMITOMO CHEM IND KK) , 19 February 1990 see abstract ---</p> | <p>1,3,8,9, 11,20-23</p> |
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International Application No

PCT/GB 95/02457

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